Expression, distribution and cellular localization of sirtuins in the normal and ischemic brain of rodent

Jayoung Kim

Department of Medical Science

The Graduate School, Yonsei University

Expression, distribution and cellular localization of sirtuins in the normal and ischemic brain of rodent

Directed by Professor Ji Hoe Heo

The Master's Thesis

submitted to the Department of Medical Science

the Graduate School of Yonsei University

in partial fulfillment of the requirements for the degree of

Master of Medical Science

Jayoung Kim

December 2015

This certifies that the Master's thesis of

Jayoung Kim is approved.

Thesis Supervisor : Ji Hoe Heo

Thesis Committee Member #1 : Young Nyun Park

Thesis Committee Member #2 : Hyun Jung Choi

The Graduate School

Yonsei University

December 2015

ACKNOWLEDGEMENTS

My life as a graduate student studying in research is finally coming to a close. As I look back on my years in finishing up my master's degree, I cannot help but be filled with big and small regrets. I would like to take this opportunity to give my sincerest thanks to all who were of huge strength to me and to those that gave me invaluable guidance and support.

First, I would like to give my deepest thanks to Professor Ji Hoe Heo in guiding my inexperienced self every step of the way and never ceasing to teach, encourage, and believe in me throughout this whole process. I am sorry that I could not present to you better results for this research as I would have liked. I would also like to give my sincerest thanks to Professor Young Nyun Park, Professor Hyun Jung Choi, and Professor Ok-Hee Lee for helping me complete my thesis with all your guidance and counseling.

Secondly, I would like to give thanks to Doctor Il Kwon, who from the time since I started working in the laboratory until the day of my conferment, generously gave me words of advice and encouragement. To Bo Hyung Lee, Sung Kyul Lee, and Yun Mi Woo, thank you for helping me in times of trouble. And to Seung-Hee Yang and Sunho An, to whom I was able to share my joys and sorrows with in the laboratory, thank you for listening to all of my troubles and worries and comforting me with your kind words.

Finally, I would like to give my warmest appreciation to my loving parents and younger brother Jong Hwi Kim who never failed to quietly pray for me and never give up on me even though there were times when I wanted to give up on myself from all the disappointments and difficulties.

This accomplishment of mine was able to come to realization because of all your kind concern and support for me. Looking back, even though there is a sense of regret, I am able to cherish it as a time where it helped me to become humble and realize my inadequacies. Through this, I will not forget my first original intention and be the best researcher that I can be.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Expression, distribution and cellular localization of sirtuins in the normal and ischemic brain of rodent

Jayoung Kim

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Ji Hoe Heo)

Sirtuins (SIRTs) are nicotinamide adenine dinucleotide-dependent histone deacetylases. To date, seven sirtuins (SIRT1-SIRT7) have been identified in mammals. While basic knowledge of sirtuins in the brain is essential to understand the role of sirtuins in the normal and pathologic brain, only limited information is available. We investigated the distribution and cellular localization of sirtuins in the normal and ischemic brain of the rodent.

Male Sprague-Dawley (SD) rats, Wistar rats, Imprinting Control Region (ICR) mice, and C57BL/6 mice were used. Normal whole brain samples were obtained. In paraffin or frozen sections, immunohistochemistry was performed and the whole brain was examined into regions: olfactory bulb, cerebrum and cerebellum. To examine the cell types that express sirtuins, double immunofluorescence staining was performed using antibodies for each sirtuin and those for specific cell markers. Cerebral ischemia induced by 2 hr of middle cerebral artery occlusion and 22 hr of reperfusion using nylon thread. The expression of each sirtuin was compared between normal and ischemic brain areas.

SIRT1, SIRT3 and SIRT5-7 were expressed in neurons of most of regions and in oligodendrocytes of corpus callosum and internal capsule. SIRT2 was expressed in myelin. SIRT4 was expressed in vessels. As a result, sirtuins, especially SIRT5-7 were widely distributed and highly expressed in the olfactory bulb, cerebrum, and cerebellum. The expression of SIRT1 and SIRT3 in the corpus callosum and internal capsule was variable among species. SIRT2 was not expressed in the external plexiform layer of olfactory bulb and hypothalamus of cerebrum. SIRT4 was widely expressed in the entire brain areas except in the purkinje layer of cerebellum in SD rats. After induction of cerebral ischemia, SIRT4 showed significantly increased expression in the ischemic region. However, the other sirtuins

were not significantly different.

Distribution and cellular localization in the rodent brain were different among sirtuins. Expression in the ischemic brain was also different. Findings of this study might provide with basic information for studying pathophysiologic role of sirtuins in the brain.

Key words : sirtuin, brain, distribution, immunohistochemistry, rodent

Expression, distribution and cellular localization of sirtuins

in the normal and ischemic brain of rodent

Jayoung Kim

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Ji Hoe Heo)

I. INTRODUCTION

Sirtuins (SIRTs) or Silent information regulator 2 (Sir2) proteins are nicotinamide adenine dinucleotide (NAD^+) - dependent histone deacetylases. Sirtuins are found from bacteria to humans and regulate crucial biological pathways in organisms.[1,2] To date, seven sirtuins (SIRT1-SIRT7) have been identified in mammals. Although sirtuins have similar structural homology, their subcellular localizations, enzymatic activities, and biological functions are different. SIRT1, SIRT6, and SIRT7 are localized in nucleus, SIRT2 is localized in cytoplasm, and SIRT3 – SIRT5 are present in mitochondria.[3-6] Sirtuins possess predominantly deacetylase activity (SIRT1-SIRT3, SIRT7), mono-ADP-ribosyltransferase activity (SIRT4, SIRT6), or desuccinylase and demalonylase activities (SIRT5).[1,7-9] Given that the subcellular localization (nucleus, cytoplasm, or mitochondria) and enzymatic activities (deacetylase or ADP-ribosyltransferase) are different among sirtuins, their distribution and cellular expression as well as roles in the brain may be different.[2,3,5,10] However, most studies have focused on SIRT1 and SIRT2. To understand and study the role and function of each sirtuin in the brain, basic knowledge on the cellular and anatomical expressions of each sirtuin in the brain is essential.

Sirtuins have been implicated in influencing a wide range of cellular processes including aging, transcription, apoptosis, inflammation and stress resistance, as well as energy efficiency and alertness during low-calorie conditions.[11,12] By virtue of their nature, sirtuins have gained interest in aging and longevity, and have been widely studied in the brain diseases such as neurodegenerative diseases and cerebral ischemia as a potential therapeutic target.[13] Ischemic

injury of the brain, which is caused by cessation of blood flow, is mediated by a variety of molecular mechanisms. Molecular mediators of cell death or survival of cerebral ischemia have been the therapeutic target. Among sirtuins, SIRT1 have been suggested as a potential therapeutic target since it is known that the inhibition of SIRT1 abolished resveratrol-induced neuroprotection.[14] As such, other sirtuins may play roles in ischemic injury. However, the effect of sirtuins in ischemic injury and the expression of sirtuins in the ischemic brain have not been well known.[15,16]

Therefore, this study investigated the distributions and cellular localization of each sirtuin in the normal brain as well as ischemic brain of the rodent to provide basis for experimental researches on sirtuins in the brain.

II. MATERIALS AND METHODS

1. Experimental animals

To examine the distributions and cellular localization of sirtuin in the normal brain, two species of male rats and two species of male mice were used. They included nine-week-old Sprague-Dawley (SD) and Wistar rats, and nine-week-old Imprinting Control Region (ICR) and C57BL/6 mice. The care and use of animals were based on the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and the protocols approved by the Animal Experiment Committee of Yonsei University College of Medicine. Rodents were housed with a controlled temperature under 12 hour light/dark cycle with free access to food and water. To obtain brain samples, under urethane anesthesia, animals were sacrificed through transcardiac perfusion with cold saline. The brain was obtained after decapitation.

2. Middle cerebral artery occlusion model and brain sampling

Cerebral ischemia was induced in SD rats. Animals were anesthetized with isoflurane (5% for induction and 2% for maintenance) in a mixture of 70% nitrous oxide and 30% oxygen. During operative procedures, body temperature was monitored continuously with a rectal probe and maintained at 37.0 ± 0.5℃ using a homeothermic blanket control unit and a heating pad (Harvard Apparatus, 507220F). After middle line incision and retraction of muscles, the left common carotid artery (CCA) was isolated. After careful dissection of vagus nerve from the CCA, CCA, occipital artery and pterygopalatine artery were isolated and ligated using a silk suture (B. Braun Medical; 5-0 Silkam®, Rubi, Spain). A commercially available 4-0 fine silicon rubber-coated nylon thread (Doccol Co., Sharon, MA, USA) was inserted after temporary clipping of the internal carotid artery (ICA) using a vascular clamp (Fine Science Tools Inc., 00396-01, North Vancouver, British Columbia, Canada). The suture was introduced 23 mm or until resistance into the left ICA through the external carotid artery (ECA) to occlude the origin of the left middle cerebral artery (MCA). After 2 hr of MCA occlusion (MCAO), reperfusion was achieved by removal of the MCAO suture. After 22 hr of reperfusion, the animals were sacrificed, and the brain was obtained.

For paraffin blocks, the extracted whole brains were fixed in 4% paraformaldehyde for overnight. Fixed brains were then sectioned into 2 mm-thick coronal blocks using a rat/mouse brain matrix and embedded in paraffin. For frozen blocks, extracted whole brains were

sectioned into 2 mm-thick coronal blocks on ice using the rat/mouse brain matrix, embedded in OCT compound (Sakura Fineteck Inc., Torrance, CA, USA), quickly frozen with 2-methylbutane cooled by dry ice and then stored at -80°C.

3. Immunohistochemical study

For immunohistochemistry, paraffin tissue sections that were cut into 4 μm-thickness were used. Antigen was retrieved IHC-Tek™ epitope retrieval solution and steamer (IHC world, Woodstock, MD, USA). After cooling from heat, sections were treated with 0.075% glycine in phosphate-buffered saline (PBS) for 10 min and with blotto (Tris-buffered saline containing 10% horse serum and 1% bovine serum albumin). After incubation with blotto, the sections were incubated with the primary antibodies for 2 hr at 37°C (SIRT4 and SIRT6) or overnight at 4°C (SIRT1 – 3, SIRT5 and SIRT7). The primary antibodies used were rabbit polyclonal anti-SIRT1 (1:1000, Millipore, 07-131, Temecula, CA, USA), rabbit polyclonal anti-SIRT2 (1:200, Sigma-aldrich, S8447, St.Louis, MO, USA), rabbit polyclonal anti-SIRT3 (1:400, Santa Cruz biotechnology Inc., sc-99143, Santa Cruz, CA, USA), goat polyclonal anti-SIRT4 (1:200, Novus Biological, NB100-1406, Littleton, CO, USA), rabbit polyclonal anti-SIRT5 (1:800, Millipore, ABE198), rabbit polyclonal anti-SIRT6 (1:300, Novus Biological, NB100-2522) and rabbit polyclonal anti-SIRT7 (1:600, Millipore, ABE103). Sections with blotto lacking primary antibodies were used as a negative control. The sections were incubated with the secondary antibodies for 30 min at 37° and then, 0.3% hydrogen peroxide in methyl alcohol for 20 min. After washing by tap water for 2 min, the sections were treated with an avidin-biotin-horseradish peroxidase complex (Vector Laboratories Ltd., Peterborough, Cambridgeshire, UK) for 30 min to amplify the signals. The signals were visualized by 3,3′-diaminobenzidine solution (PBS containing 0.05% 3,3′-diaminobenzidinetetrahydrochloride hydrate and 0.1% hydrogen peroxide). After counterstaining with hematoxylin, the slides were mounted with permount mounting medium (Fisher Scientific, Fair lawn, NJ, USA).

4. Immunofluorescence staining

To examine the cell types that express each of sirtuins, brain sections of SD rats were double-stained with antibodies against each sirtuin and specific cell markers. The staining procedures were similar to those in immunohistochemistry, but concentrations of antibodies for some sirtuins were slightly changed (1:100 for SIRT3, 1:500 for SIRT5, and 1:100 for SIRT7). The antibodies for cell markers used were mouse monoclonal anti-NeuN (1:100, Millipore, MAB377) or mouse monoclonal anti-β-tubulin Ⅲ (1:20, Abcam, Ab7751) for neurons, goat polyclonal anti- Glial Fibrillary Acidic Protein (GFAP) (1:200, Santa Cruz biotechnology Inc.,

sc-6170) for astrocytes, mouse anti-collagen type IV (1:100, Developmental studies hybridom bank, M3F7, Iowa City, IA, USA) or anti-rat endothelial cell antigen-1 (RECA-1, 1:10, Abcam, ab9774, Cambridge, Cambridgeshire, UK) for microvessels, mouse anti-CNPase (1:400, Abcam, ab6319) for oligodendrocytes, and mouse monoclonal anti- Myelin basic protein (MBP) (1:1000, Covance, SMI-99P, Madison, WI, USA) for myelin. In case of double-staining with SIRT4 antibody, rabbit polyclonal anti-GFAP (1:200, Millipore, AB5804) was used. To stain the brain tissue with anti-SIRT4 and anti-RECA-1 antibodies, the 10-μm thick frozen sections were used. For the remaining staining, paraffin sections were used. To visualize the signals, the sections were incubated in FITC-labeled anti-rabbit IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA) and either Cy3-labeled anti-goat IgG (Jackson Immunoresearch Laboratories) or Cy3-labeled anti-mouse IgG (Jackson Immunoresearch Laboratories) for 30 min in the dark condition. After the incubation and wash with PBS, the sections were mounted in Vectashield Hard Set™ mounting medium with DAPI (Vector Laboratories Inc., Burlingame, CA, USA).

5. Examined brain areas for determination of sirtuin expression

To address the distribution of each sirtuin, whole rodent brains were classified into olfactory bulb, cerebrum, and cerebellum. In the olfactory bulb, glomerular layer, external plexiform layer, granular cell layer, and anterior olfactory nucleus were identified. Expression of each sirtuin in the cerebrum was examined in eight representative regions: cerebral cortex, hippocampus, corpus callosum, internal capsule, caudate putamen (striatum), thalamus, hypothalamus, and amygdala. In the cerebellum, molecular layer, purkinje cell layer, granular layer, and deep cerebellar nuclei were included (Fig. 1).

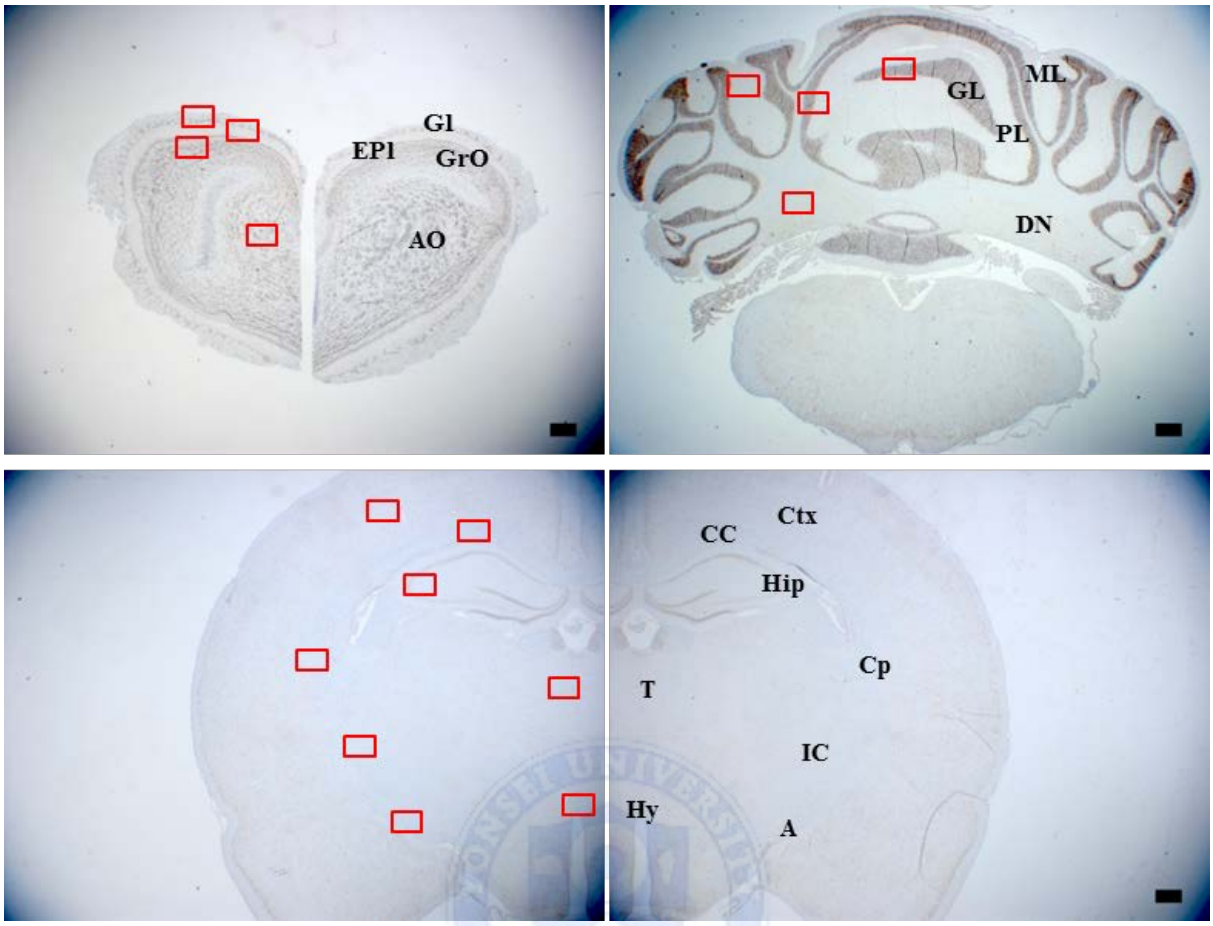


Figure 1. Representative regions of olfactory bulb, cerebrum, and cerebellum of the rodent brain. Gl : Glomerular layer, EPl : Expernal plexiform layer, GrO : Granular cell layer, AO : Anterior olfactory nucleus, Ctx : Cerebral cortex, Hip : Hippocampus, CC : Corpus callosum, IC : Internal capsule, Cp : Caudate Putamen, T : Thalamus, Hy : Hypothalamus, A : Amygdala, ML : Molecular layer, PL : Purkinje cell layer, GL : Granular layer, DN : Deep cerebellar nuclei. Scale bars represent 500㎛.

6. Analysis

The consistency of immunostaining was ensured by triplication of experiments by using three rats or mice of each species. We investigated whether there is any difference in the expression and distribution of sirtuins between the rat and mouse and also among the species. Sections were viewed using Axio Imager D2 microscope and Axio Vision software (Carl Zeiss MicroImaging GmbH, Jena, Germany) or using LSM700 scanning laser confocal microscope and Zen 2011 image software (Carl Zeiss MicroImaging GmbH, Jena, Germany).

To determine the expression pattern of sirutins, we measured and compared cell numbers

between ischemic regions and nonishemic regions based on immunohistochemical staining for each sirtuins in the brain of SD rats (n=5). The percentage of sirtuin- positive cells were estimated by stereological analysis with Stereo investigator software (MBF bioscience, Williston, VT, USA) using an optical fractionator. To obtain an unbiased number of positive cells of each sirtuin, all contours around ischemic core were traced under 12.5 x magnification. Counting frame size was set at 200 x 200 um, and the sampling grid size at 900 x 900um. Random placement of the counting frames was accomplished using the motorized stage of microscope controlled by the software. The numbers of cells in the counting frame were counted at 400 x magnification. Cell density was expressed as the number of cells per unit area (mm^2). To compare the cell number between ischemic and nonischemic areas, the mean and standard error (SE) were calculated and independent-sample *t* tests were used. Statistical analysis was performed using Prism 6 for Windows (Version 6.07, GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

III. RESULTS

1. Distribution of each sirtuin in the brain

We found that all sirtuins were expressed in the brain of rats and mice. However, their distribution was different among sirtuins (Fig. 2). In addition, there were some differences in the cellular and anatomical expressions of each sirtuin between species (Table. 1).

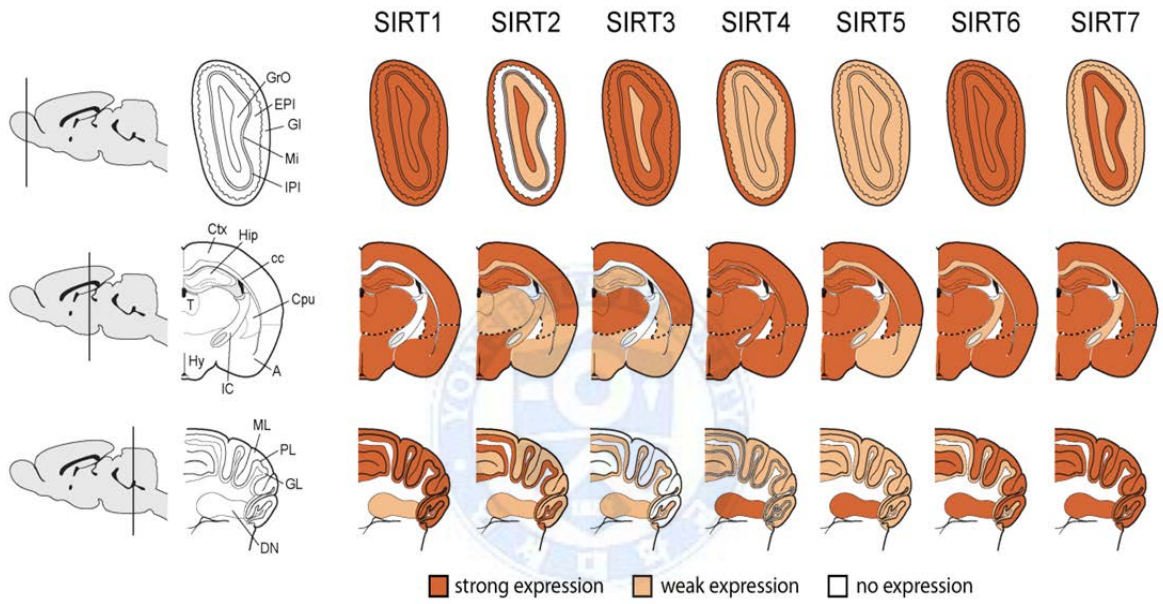


Figure 2. Representative cartoon of each sirtuin in the brain of SD rats. This cartoon shows each expression level of sirtuins in the specific regions of olfactory bulb (upper), cerebrum (middle), and cerebellum (lower) through sirtuins. Gl : Glomerular layer, EPl : Expernal plexiform layer, Mi : Mitral cell layer, GrO : Granular cell layer, IPl : Internal plexiform layer, AO : Anterior olfactory nucleus, Ctx : Cerebral cortex, Hip : Hippocampus, CC : Corpus callosum, IC : Internal capsule, Cp : Caudate Putamen, T : Thalamus, Hy : Hypothalamus, A : Amygdala, ML : Molecular layer, PL : Purkinje cell layer, GL : Granular layer, DN : Deep cerebellar nuclei.

Table 1. Expression of sirtuins in the representative regions of the brain in rodents

		Sirt1	Sirt2	Sirt3	Sirt4	Sirt5	Sirt6	Sirt7
Olfactory bulb	Glomerular layer (Gl)	+	+	+	+	+	+	+
	External plexiform layer (EPl) [including mitral cell layer (Mi)]	+	+/-	+	+	+	+	+
	Granular cell layer (GrO) [including internal plexiform layer (IPl)]	+	+	+	+	+	+	+
	Anterior olfactory nucleus (AO)	+	+	+/-	+	+	+	+
Cerebrum	Cerebral cortex (Ctx)	+	+	+	+	+	+	+
	Hippocampus (Hip)	+	+	+	+	+	+	+
	Corpus callosum (CC)	+/-	+	+/-	+	+	+	+
	Internal capsule (IC)	+/-	+	+/-	+	+	+	+
	Caudate Putamen (Cp)	+	+	+	+	+	+	+
	Thalamus (T)	+	+	+	+	+	+	+
	Hypothalamus (Hy)	+	+/-	+	+	+	+	+
	Amygdala (A)	+	+	+	+	+	+	+
Cerebellum	Molecular layer (ML)	+	+	+/-	+	+	+	+
	Purkinje cell layer (PL)	+	+	+	+/-	+	+	+
	Granular layer (GL)	+	+	+	+	+	+	+
	Deep cerebellar nuclei (DN)	+	+	+	+	+	+	+

+ indicates the presence of signals. +/- means that some species show the signal, but other species do not.

SIRT1 – no detection in the corpus callosum and internal capsule of SD rats and ICR mice

SIRT2 – no detection in the external plexiform layer of SD rat and in the hypothalamus of ICR mice

SIRT3 – no detection in the anterior olfactory nucleus of Wistar rats and C57BL/6 mice and in the corpus callosum, internal capsule and molecular layer of SD rats

SIRT4 – no detection in the purkinje cell layer of SD rats

SIRT1

SIRT1 was widely distributed and highly expressed in the olfactory bulb, cerebrum, and cerebellum, except for the corpus callosum and internal capsule in all examined rodents. The expression was less intense in the deep cerebellar nuclei (Fig. 3). Expression of SIRT1 in the corpus callosum and internal capsule showed variation between species in that SIRT1 was expressed in Wistar rats and C57BL/6 mice, but not in SD rats and ICR mice.

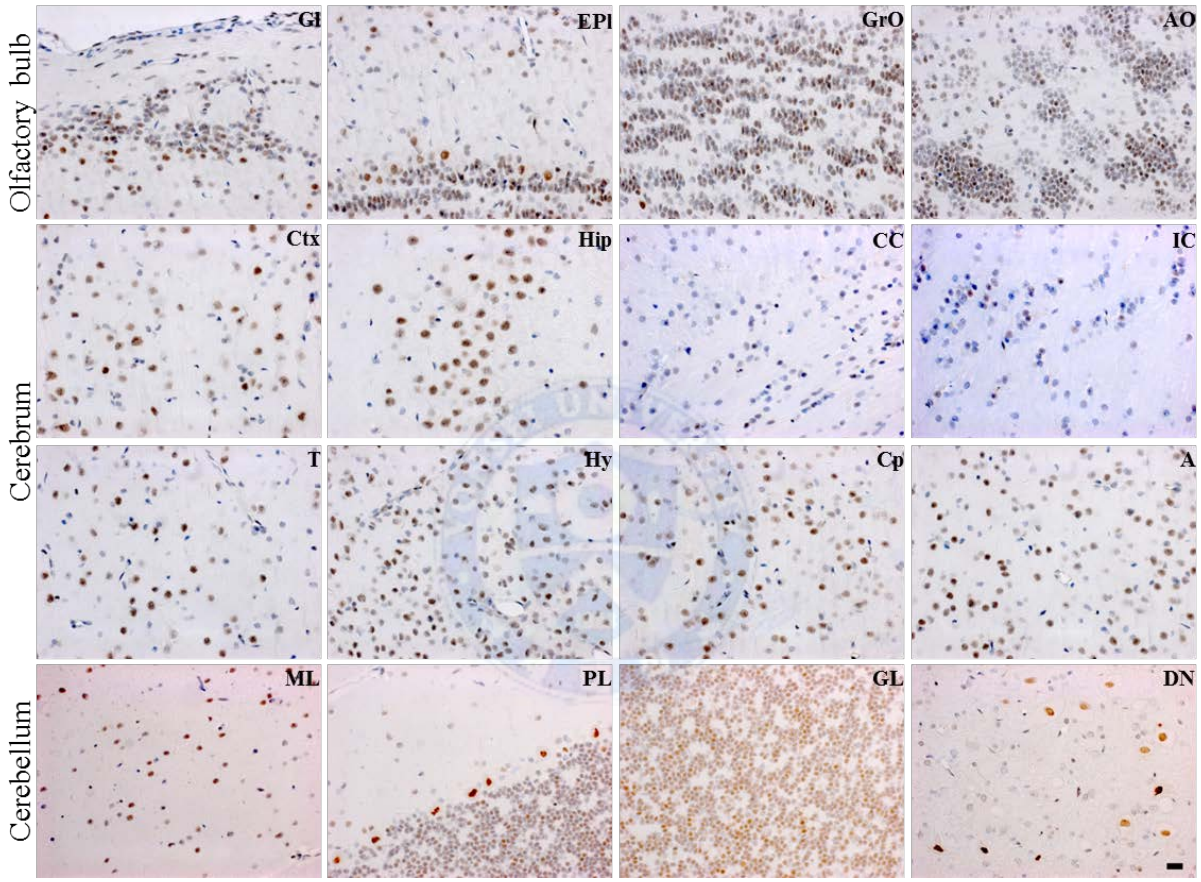


Figure 3. Representative expression pattern of SIRT1 in the brain of SD rats. SIRT1 is expressed in the all examined brain regions except for the corpus callosum and internal capsule. Scale bar represents 20㎛.

SIRT2

SIRT2 protein was detected in most regions of the olfactory bulb in mice and rats. However, SIRT2 was not expressed in the external plexiform layer of SD rats. SIRT2 was expressed in the all examined areas of cerebrum and cerebellum except for ICR mice that did not express SIRT2 in the hypothalamus (Fig. 4).

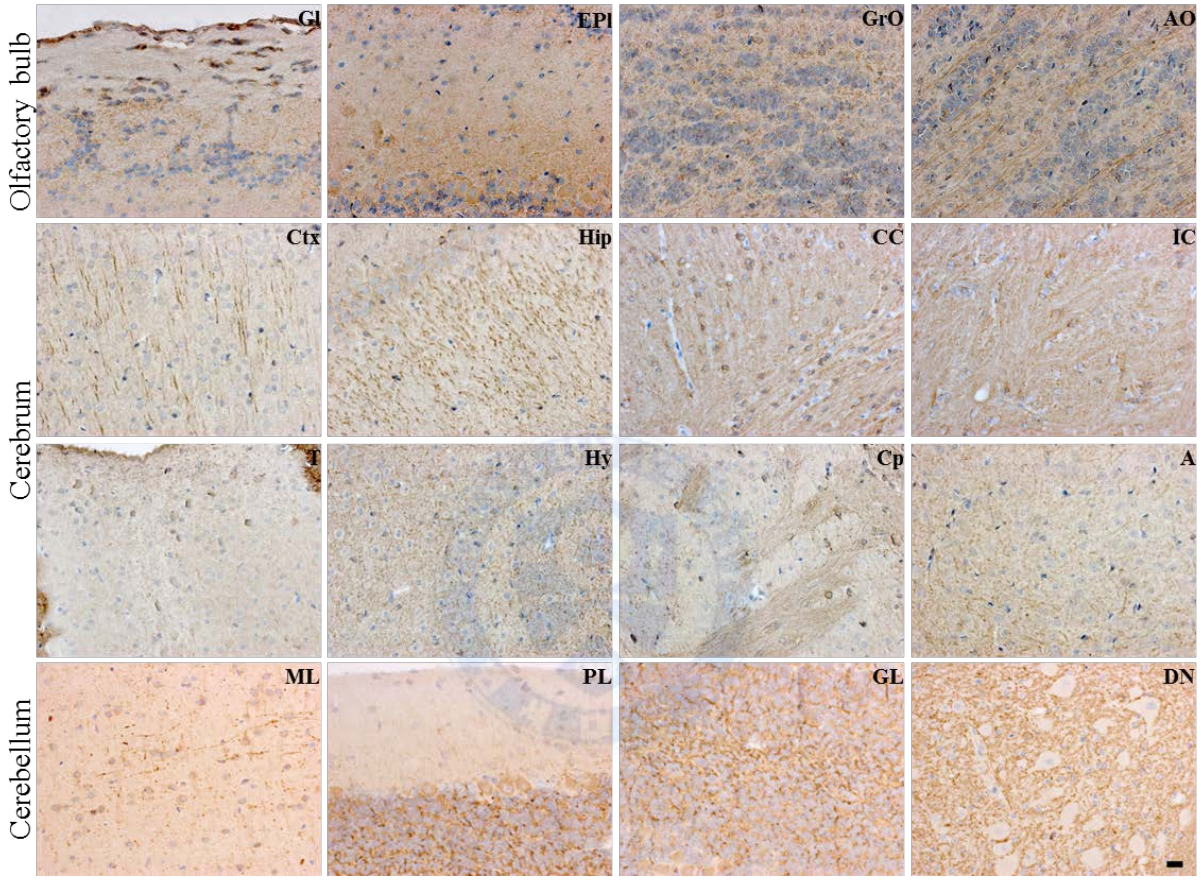


Figure 4. Representative expression pattern of SIRT2 in the brain of SD rats. Most regions show SIRT2-positive signals except for the external plexiform layer of olfactory bulb. Scale bar represents 20㎛.

SIRT3

SIRT3 was widely distributed in the olfactory bulb. However, SIRT3 was not expressed in the anterior olfactory nucleus of Wistar rats and C57BL/6 mice. SIRT3 was widely expressed in the all examined areas of the cerebrum and cerebellum in Wistar rats, ICR mice, and C57BL/6 mice. However, SIRT3 was not expressed in corpus callosum, internal capsule and molecular layer of cerebellum in SD rats (Fig. 5).

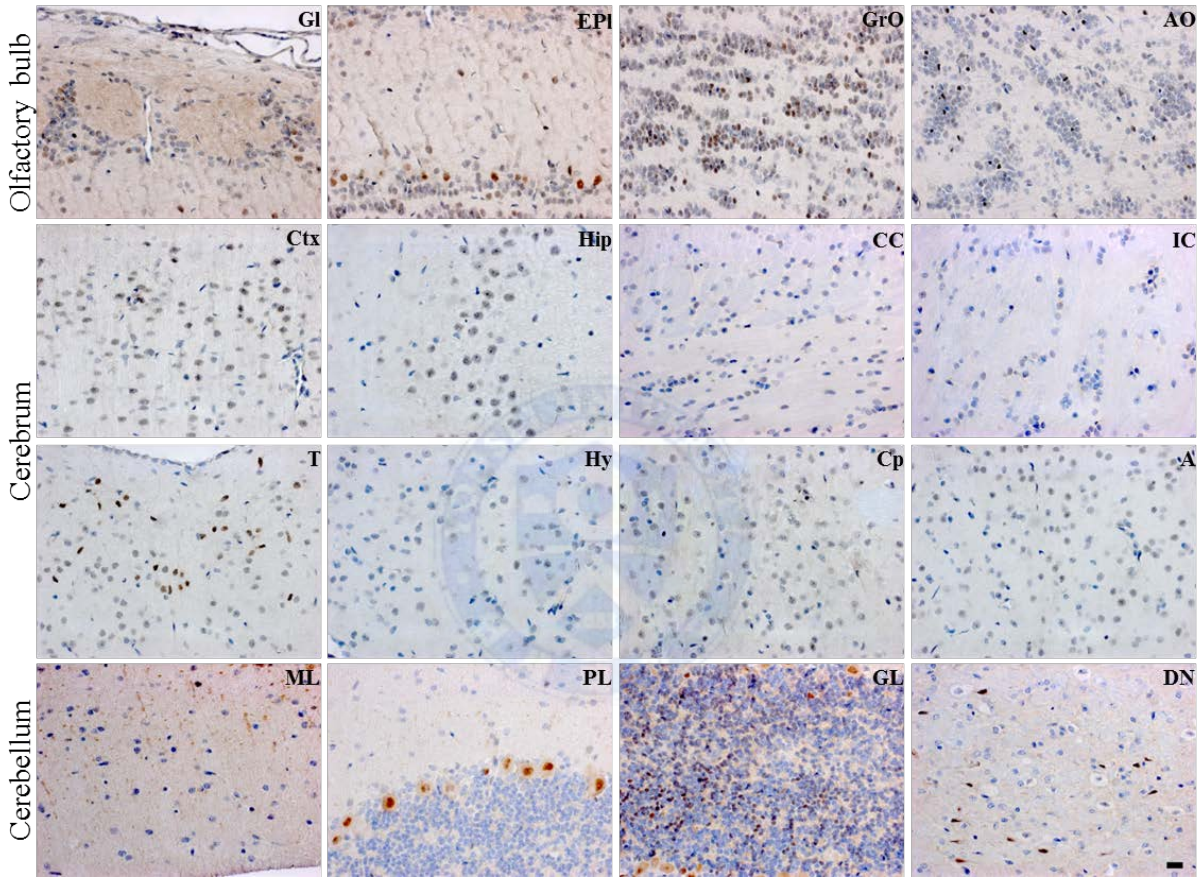


Figure 5. Representative expression pattern of SIRT3 in the brain of SD rats. SIRT3 was widely distributed in the olfactory bulb and the cerebrum. However, SIRT3 was not expressed in corpus callosum, internal capsule and molecular later of cerebellum. Scale bar represents 20㎛.

SIRT4

SIRT4 protein could be found in the whole olfactory bulb, cerebrum and cerebellum. However, it was hard to find positive signals in the purkinje cell layer of SD rats (Fig. 6).

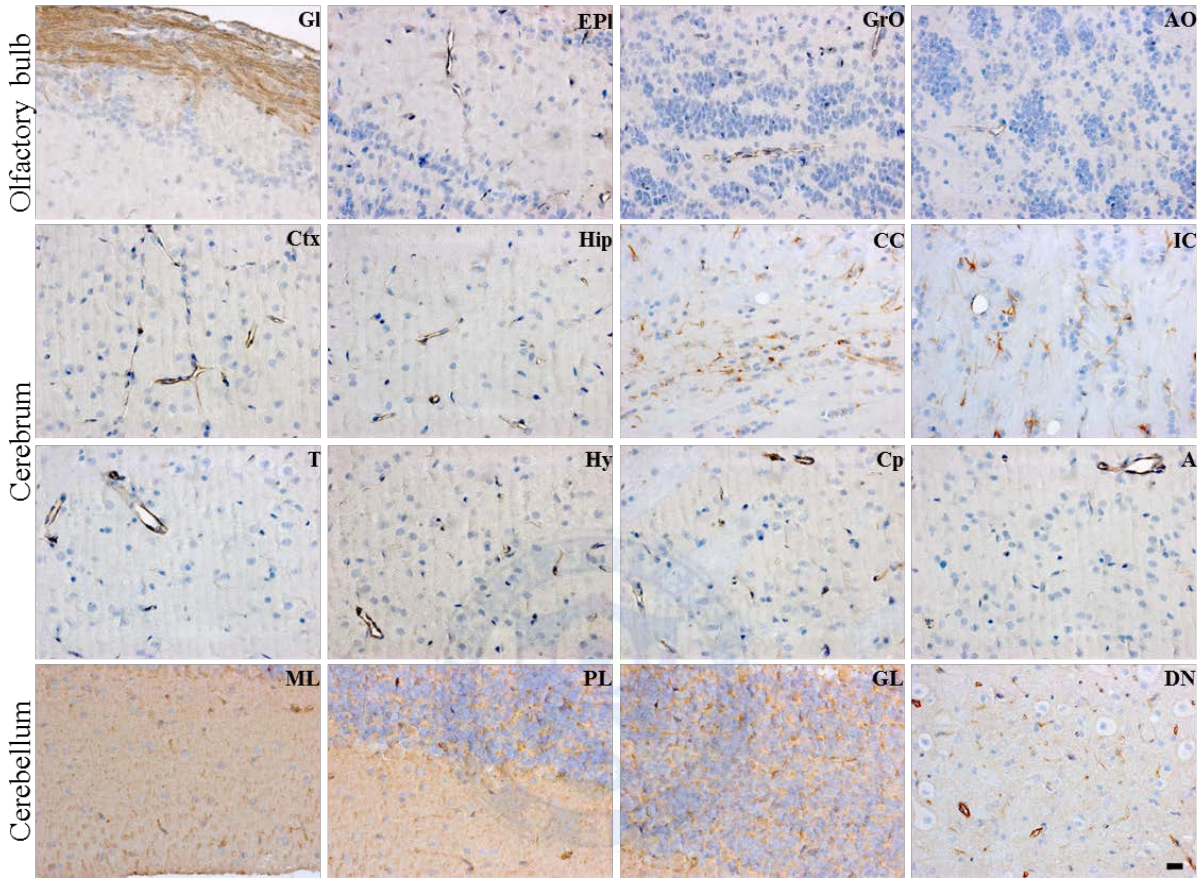


Figure 6. Representative expression pattern of SIRT4 in the brain of SD rats. SIRT4 is detected in whole olfactory bulb and cerebrum of the rodent brain. SIRT4 also show positive signals in the cerebellum but it is hard to find certain signals in the purkinje cell layer of cerebellum. Scale bar represents 20㎛.

SIRT5

SIRT5 was weakly expressed in the olfactory bulb when comparing to other sirtuins. As well, SIRT5 was sparsely expressed in the glomerular layer. However, SIRT5 was strongly expressed in the all examined areas of cerebrum. While SIRT5 was expressed in the entire cerebellum, its expression was stronger in the purkinje cell layer and deep cerebellar nuclei than in the molecular layer and granular layer (Fig. 7).

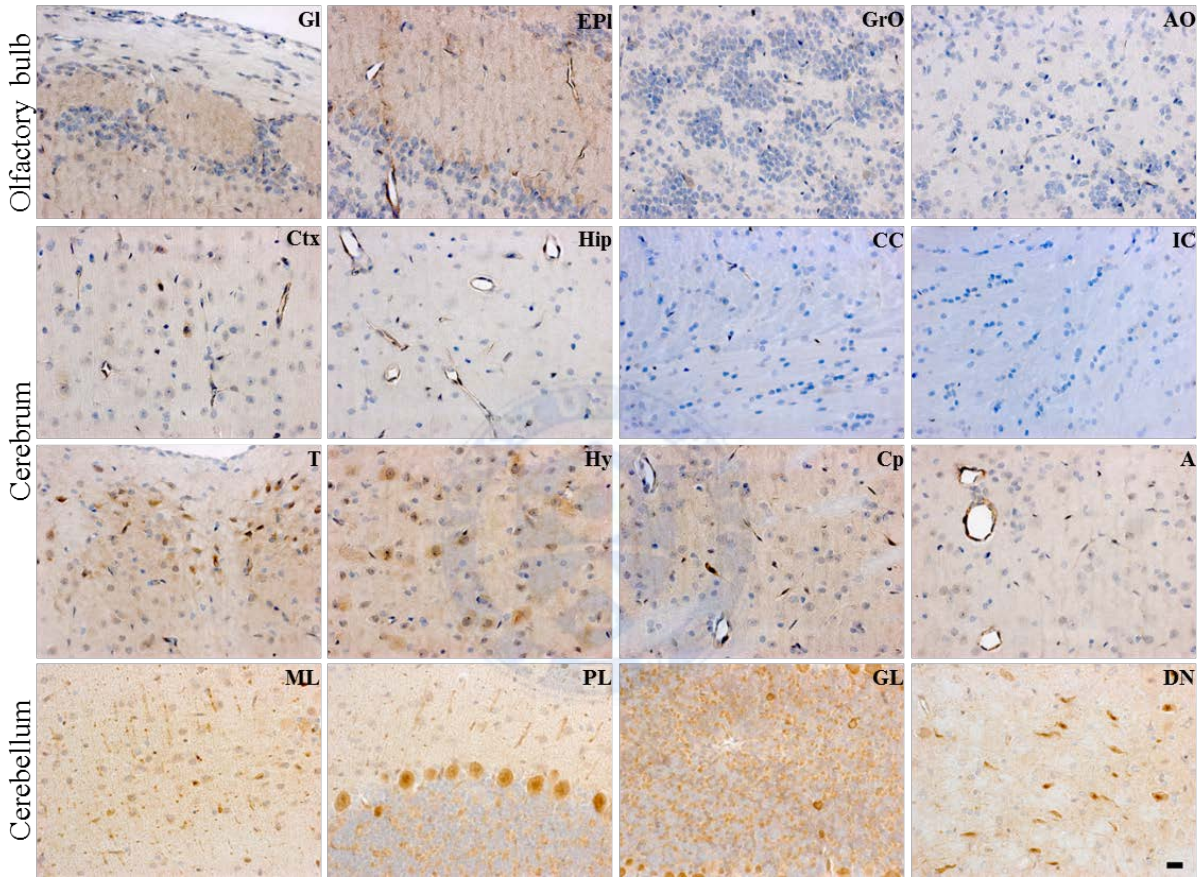


Figure 7. Representative expression pattern of SIRT5 in the brain of SD rats. SIRT5 was expressed in the all examined areas of olfactory bulb, cerebrum and cerebellum. Scale bar represents 20㎛.

SIRT6

SIRT6 protein showed wide distribution and strong expression throughout the entire regions of rodent brain (Fig. 8).

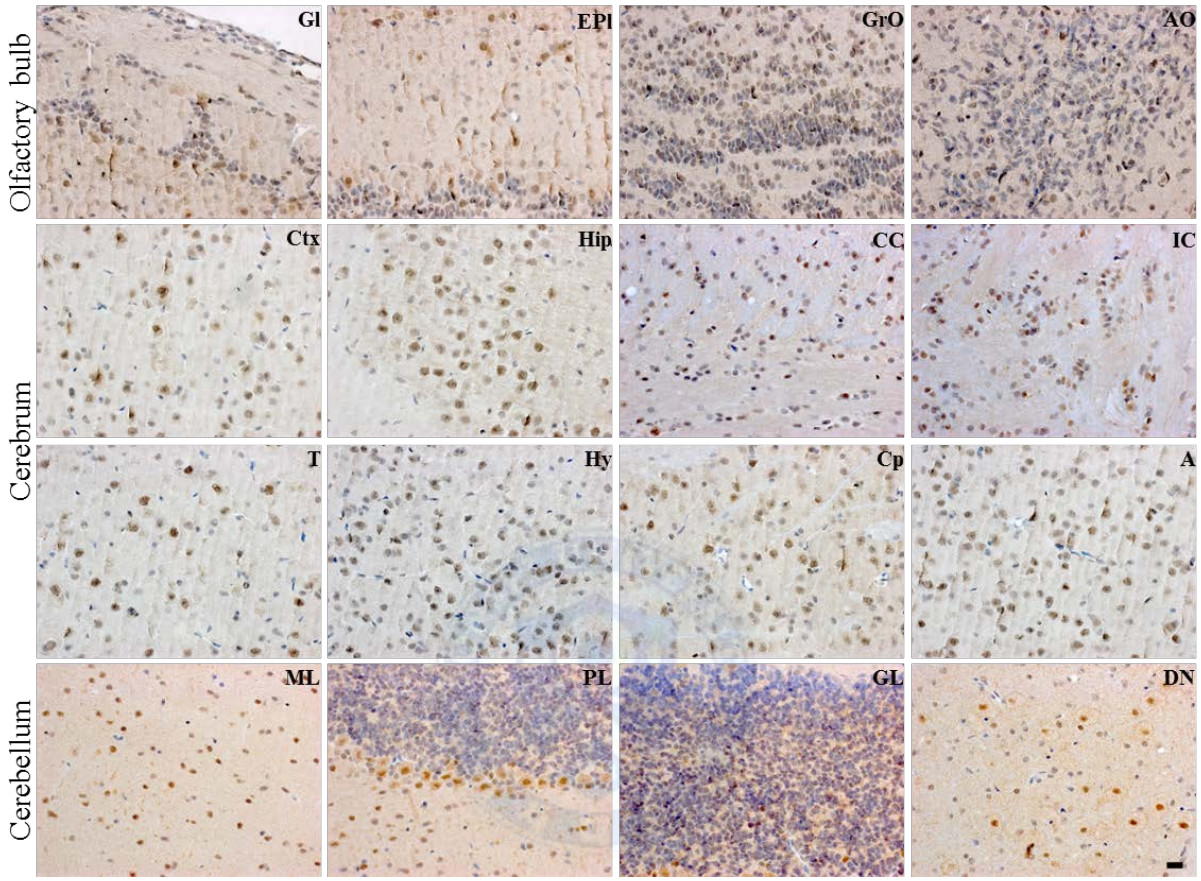


Figure 8. Representative expression pattern of SIRT6 in the brain of SD rats. SIRT6 is highly expressed in all representative regions of the rodent brain. Scale bar represents 20㎛.

SIRT7

SIRT7 was widely expressed in the olfactory bulb as well as in the all regions of the cerebrum and cerebellum with high intensity. (Fig. 9).

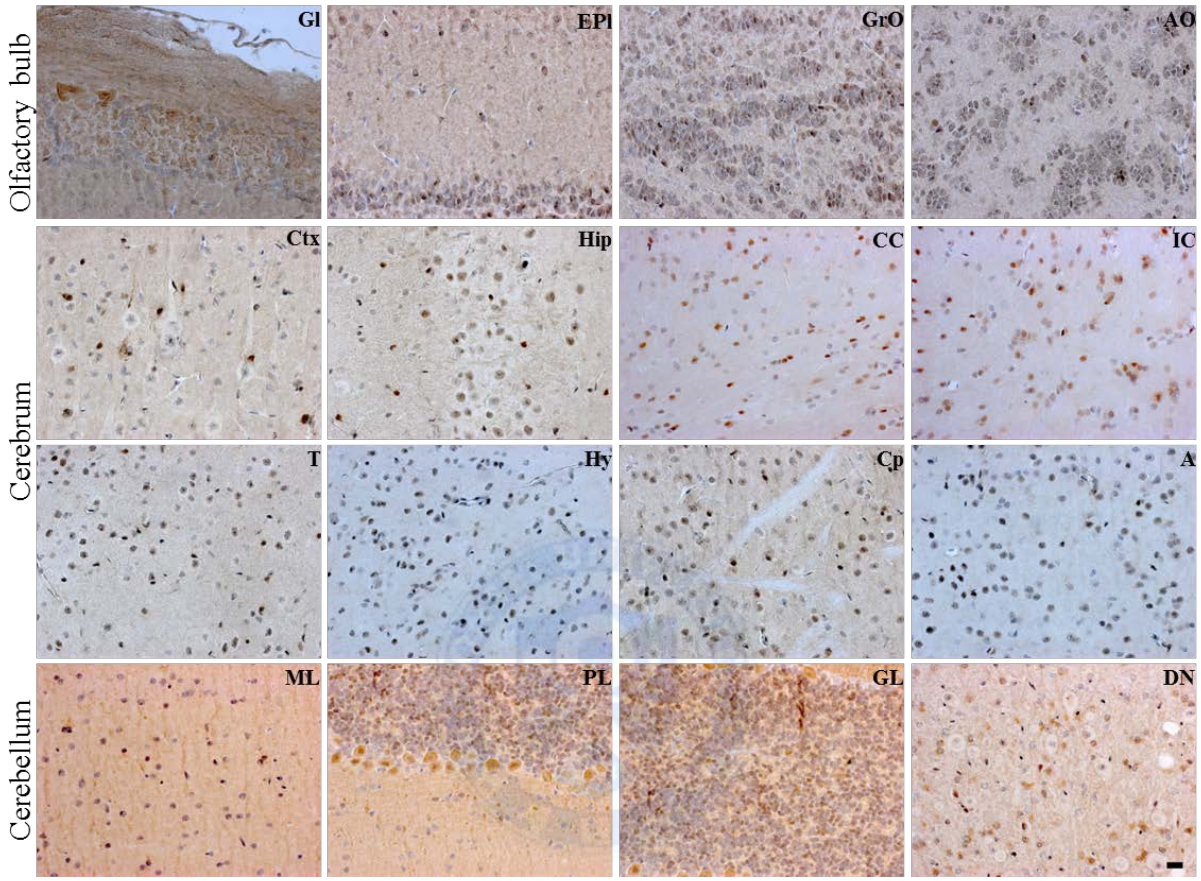


Figure 9. Representative expression pattern of SIRT7 in the brain of SD rats. SIRT7 is broadly distributed in the rodent brain. Most regions show high expression level of SIRT7. Scale bar represents 20㎛.

2. Cellular localization of each sirtuin

Cellular localization was determined using dual immunofluorescence staining in the brain of SD rats. SIRT1 was co-localized with NeuN-positive cells, which suggests that SIRT1 was expressed in neuronal cells (Fig. 10A). SIRT2 was not co-localized with anti-NeuN, anti-GFAP or anti-Collagen type antibody Ⅳ. Expression pattern of SIRT2 was filamentous-, and co-localized with MBP-positive cells, suggesting that SIRT2 was present in myelin (Fig. 10B). SIRT3 was not co-localized with anti-NeuN but with anti-β-Ⅲ-tubulin antibody that is specific for all neurons (Fig. 10C). This finding suggests that SIRT3 was associated with neuronal cells. Unique morphology of SIRT4 was verified by staining with anti-collagen type Ⅳ or anti-RECA-1. SIRT4 signals were co-localized with collagen type Ⅳ-positive cells and RECA-1-positive cells, implying that SIRT4 was expressed in vessels (Fig. 10D).

We also found that NeuN-positive cells were co-expressed with SIRT5-7 proteins (Fig. 11). Collagen type Ⅳ-positive cells were co-localized with some SIRT5 proteins (Fig. 11B). SIRT5-7 showed some signals in the corpus callosum and internal capsule where neuronal cells do not present, and were co-localized with oligodendrocytes on dual staining with CNPase antibody (Figure 11D). These findings suggest that SIRT5-7 are expressed in neurons and oligodendrocytes. All seven sirtuins were not detectable in GFAP-positive cells, which suggest that sirtuins are not expressed in astrocytes (Table. 2).

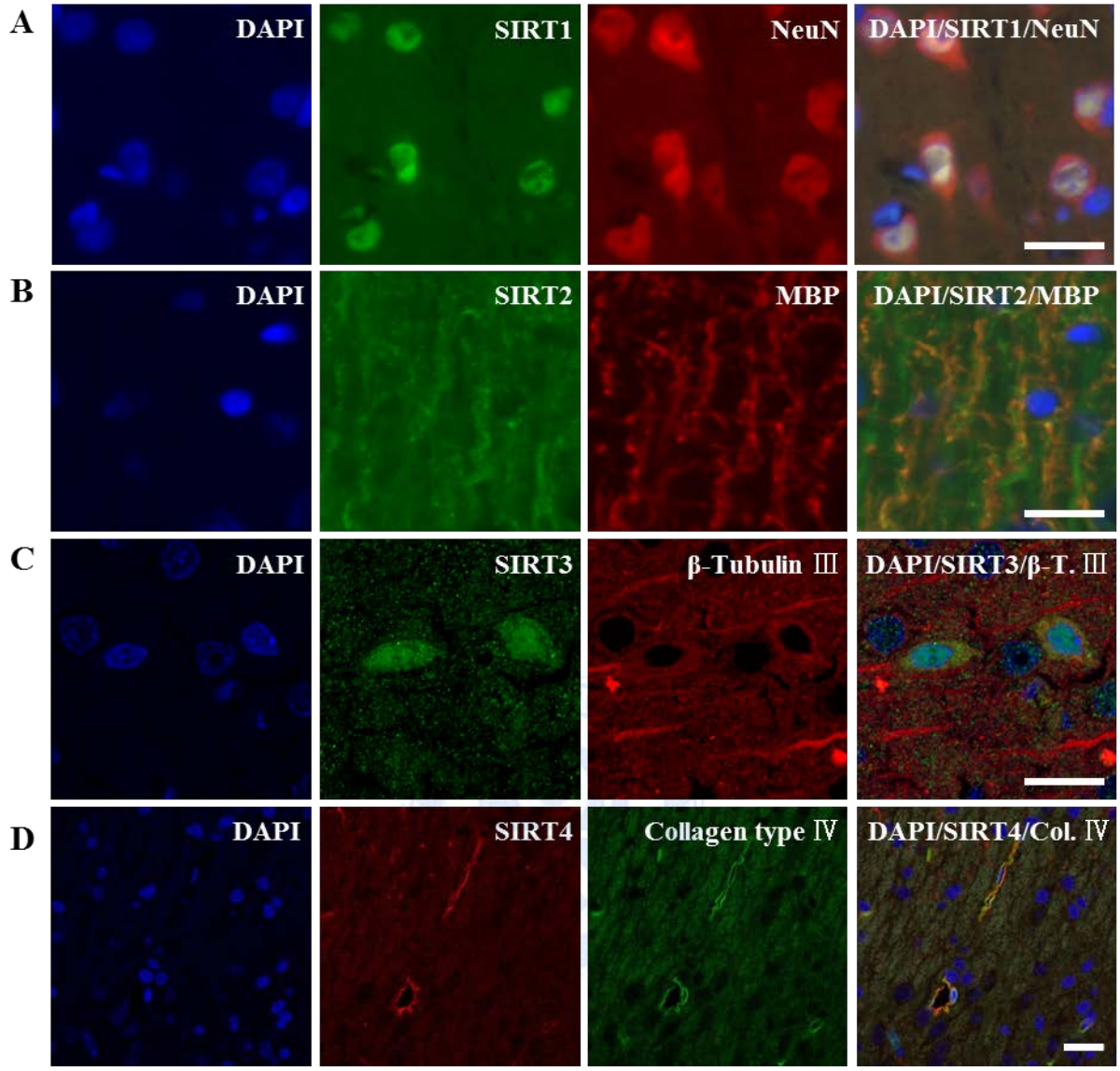


Figure 10. Cellular localization of SIRT1-4 in SD rats. (A) SIRT1 is co-localized with Neuronal nuclei (NeuN). (B) SIRT2 is merged with myelin basic protein (MBP). (C) SIRT3 is partially co-localized with NeuN but mostly with β-tubulin Ⅲ (β-T.Ⅲ). (D) SIRT4 is co-localized with collagen type Ⅳ or Rat endothelial cell antigen-1 (RECA-1). SIRT1 and SIRT3 are partially co-localized with CNPase. Scale bar represents 20㎛.

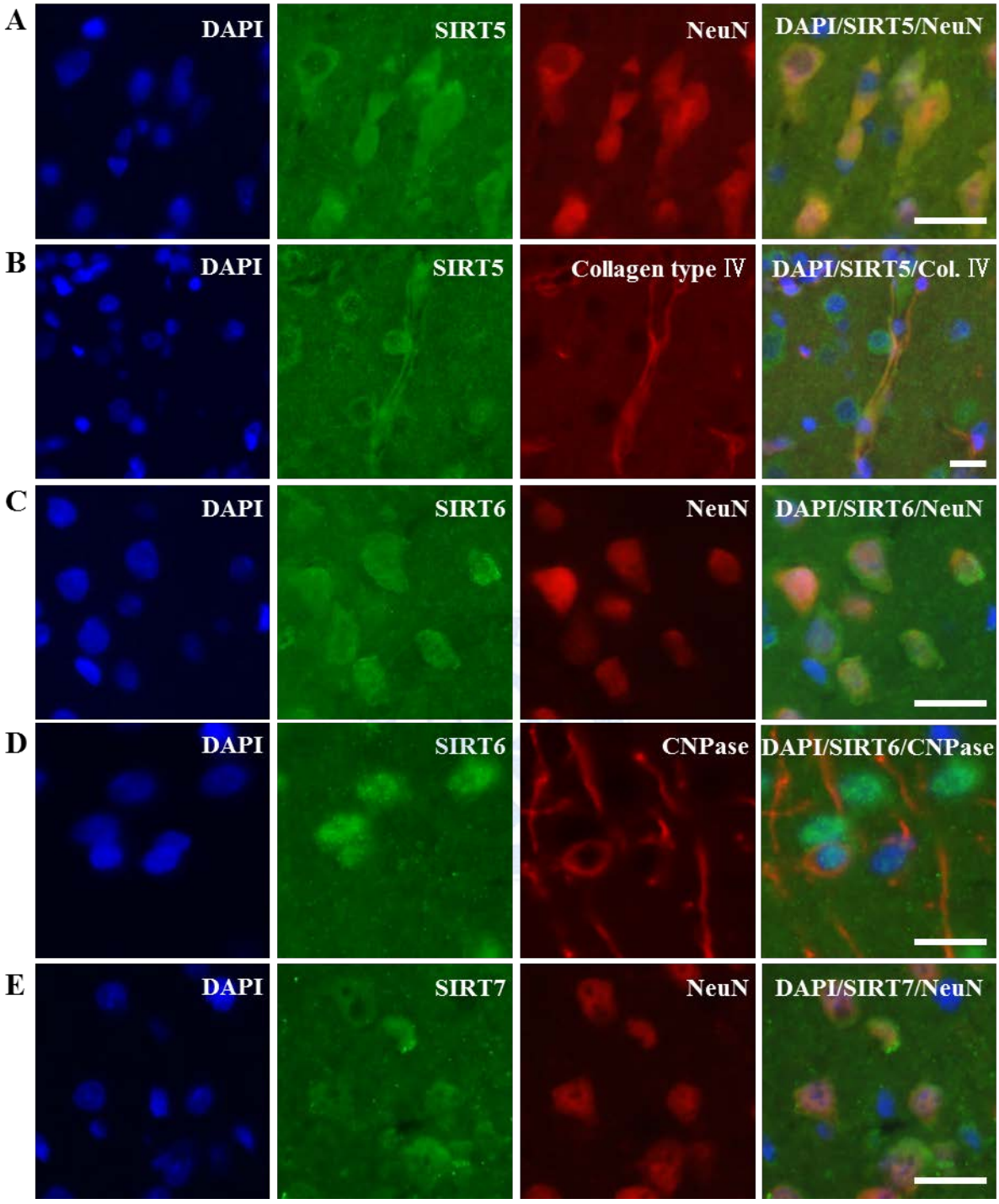


Figure 11. Cellular localization of SIRT5-7 in SD rats. (A, B) SIRT5 mainly co-localized with neuronal cells and partially with collagen type Ⅳ. (C, E) SIRT6 and SIRT7 were mainly expressed in neuronal cells. (D) SIRT6 including SIRT5 and SIRT7 partially co-localized with CNPase. Scale bar represents 20㎛.

Table 2. Examination of co-localization between each sirtuin and specific marker in the brain of SD rats

Cellular localization	SIRT1	SIRT2	SIRT3	SIRT4	SIRT5	SIRT6	SIRT7
*** Neuronal cell**	+	-	+	-	+	+	+
*** Blood vessel**	-	-	-	+	+	-	-
Myelin	-	+	-	-	-	-	-
Astrocyte	-	-	-	-	-	-	-
Oligodendrocyte	-	+	-	-	+	+	+

+ : co-localized between each sirtuin and specific marker

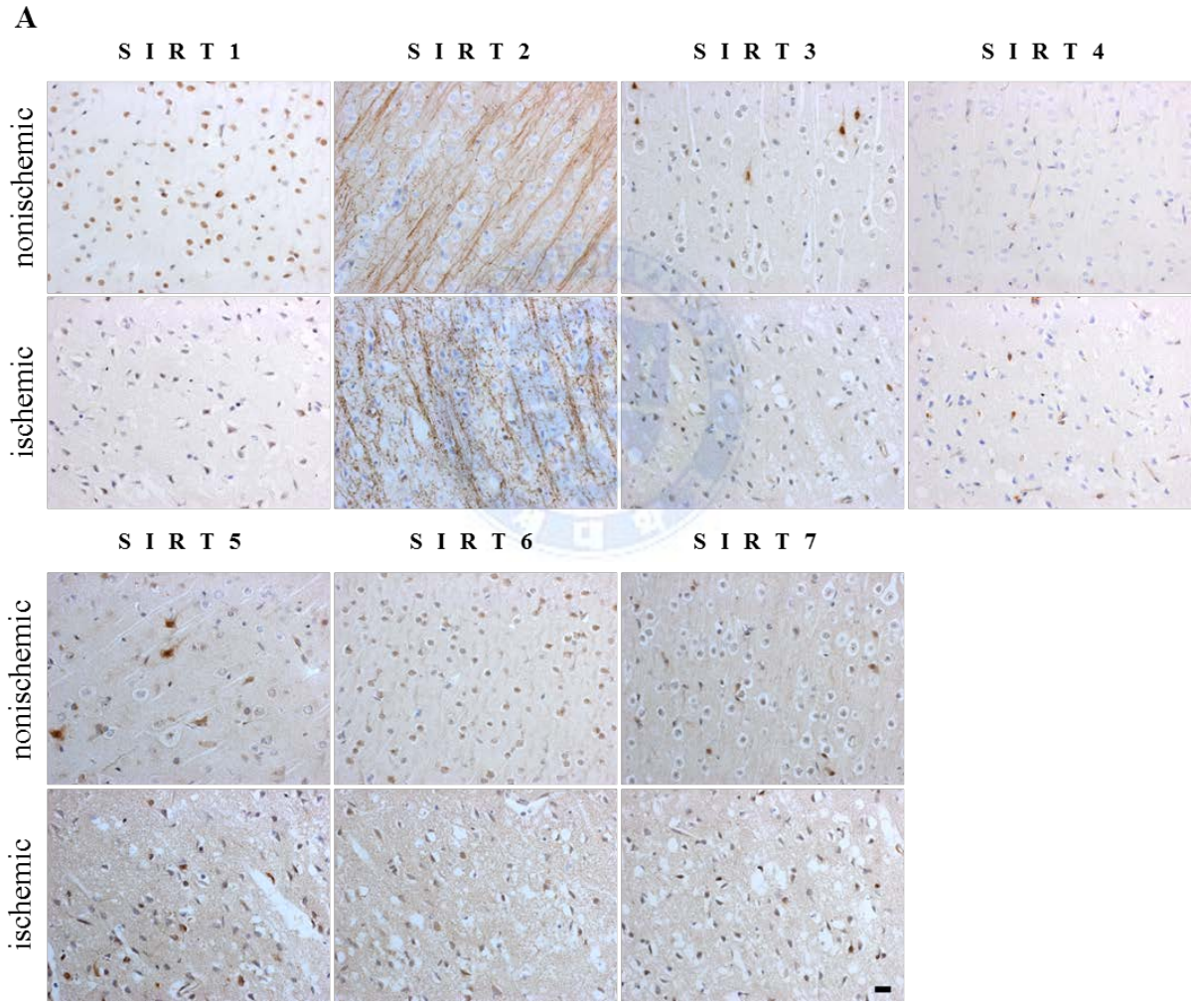
- : not merged between each sirtuin and specific marker

*Two types of neuronal cell marker were used (NeuN, β-tubulin Ⅲ). Cell markers for blood vessel were also two different antibodies (Collagen type Ⅳ, RECA-1).

Myelin (MBP), Astrocyte (GFAP), Oligodendrocytes (CNPase)

3. Expression pattern and cell density of each sirtuin after cerebral ischemia

The number of cells that express sirtuins in the ischemic area was compared those in the corresponding nonischemic area (Fig. 12A). The cell number was counted and compared in all sirtuins but SIRT2 that is expressed in myelin. The number of SIRT1, SIRT6, or SIRT7-positive cells tended to be decreased in the ischemic region. The number of SIRT3 or SIRT5-positive cells tended to be increased in the ischemic region. Quantification revealed that the number of SIRT4-positive cells was significantly higher in the ischemic region than in the nonischemic region (P=0.019) (Fig. 12B).



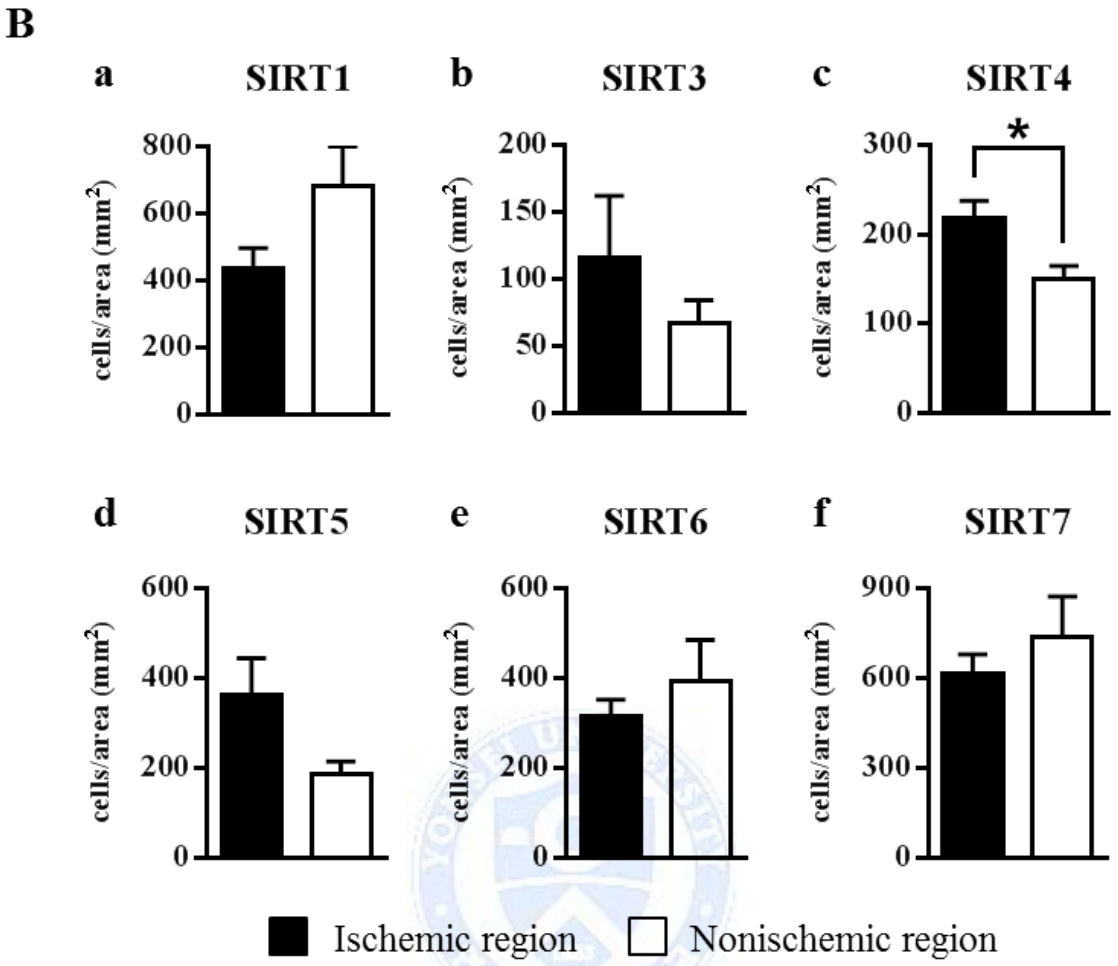


Figure 12. Cell density for each sirtuins. Using Stereo investigator program, the number of cells were measured based on immunohistochemical staining for each sirtuin. The number of SIRT4 was significantly higher in the ischemic region than the nonischemic region (P=0.019) (c). The cell density was not significantly different in the other sirtuins (a, b, d, e, f). The cell number could not be counted in SIRT2 because it expresses in myelin.

IV. DISCUSSION

In this study, we demonstrated that sirtuins are highly and widely expressed in the rodent brain across the species/strain at protein levels. However, their expression patterns including regional distribution and cellular localization were different among sirtuins. Our findings also suggested that the expression of sirtuins were slightly different among each species/strain of the rodent. As such, these strain and species-specific differences should be considered for researches using sirtuins in the rodent brain.

Rodents have developed olfactory structures unlike human because rodents use scent marking in many social contexts. All regions of olfactory structures are highly organized and composed of many layers. All sirtuins were detected throughout olfactory bulb of rodents except SIRT2 and SIRT3 that showed variation between species. SIRT2 was not expressed in the external plexiform layer of SD rats, and SIRT3 was not in the olfactory nucleus of the Wistar rats and C57BL/6 mice. The external plexiform layer has important roles in the second level of synaptic processing in the olfactory bulb between the glomerular layer and the mitral cell layer. The anterior olfactory nucleus is likely to play a central role in olfactory information processing. In this study, sirtuins were strongly expressed in the glomerular layer of the olfactory bulb which is an important way station in the pathway from the nose to the olfactory cortex and is critical for odorant signal transduction. It suggests that sirtuins might contribute to synaptic procession of odor information coming from the nose.

The cerebrum is the largest and most developed part of the brain. Sirtuins were highly expressed in the cerebral cortex regions as well as hippocampus, thalamus and hypothalamus of rodent brain. Expression of SIRT1 and SIRT3 in the corpus callosum and internal capsule showed variation between species. The corpus callosum and internal capsule are large white matter structures in the brain. They are composed of axons and glial cells, while neuronal somata make up less than 1%. SIRT1 and SIRT3, which were mainly detected in neuronal cells, were also expressed in the corpus callosum and internal capsule in Wistar rats and C57BL6 mice.

In this study, in contrast to other rodents, SIRT2 was not detected in the hypothalamus of ICR mice. SIRT2 might be involved in maintaining cellular ATP levels.[17] Decreased ATP levels in SIRT2 knock-out mice could lead to activation of the AMPK pathway, which in turn has been shown to affect multiple downstream events including hypothalamic inhibition of proopiomelanocortin.[18] However, we could not infer any answer about the absence of SIRT2 in the hypothalamus of ICR mice.

In case of the cerebellum, all sirtuins were distributed in four representative regions of cerebellum, while SIRT3 and SIRT4 were not in the particular regions of SD rats. At the top lies the molecular layer, which contains the flattened dendritic trees of Purkinje cells. It shows that molecular layer and purkinje cell layer are connected to each other for processing. SIRT3 and SIRT4 protein levels are known to have modestly lower expression levels in the cerebellum than other regions such as cortex, hippocampus, and striatum of Wistar rat brain.[19] This study showed that the diversity of expression levels in SIRT 3 and 4 were more variable in SD rats than Wistar rats. Consequently, it was hard to find expression of SIRT3 and SIRT4 in the cerebellum of SD rats. Moreover, SIRT3 and SIRT4, the mitochondrial sirtuins, may respond to changes in cellular and nutrient stress by modification of downstream target proteins.[20,21]

This study did not investigate the role or function of each sirtuin in the brain. However, the different expression pattern of sirtuins in the brain in terms of distribution in the brain, localization in the brain cells, and inherent activities suggests the diverse roles of sirtuins in the brain. SIRT1 and SIRT5-7 were mainly localized in neuronal cells. SIRT1 is known to be ubiquitously expressed across the entire rodent brain, and to be predominantly localized in neuronal nuclei, which is consistent with findings of this study. SIRT1 has been suggested to promote axonal elongation, neurite outgrowth, and dendritic branching. SIRT1 also plays protective roles in several neurodegenerative diseases including Alzheimer's, Parkinson's, and motor neuron diseases, which may relate to its functions in metabolism, stress resistance, and genomic stability.[22] SIRT3 mediates the caloric reduction of oxidative DNA damage in multiple tissues.[23] These findings suggest that SIRT1 and SIRT3 play an important role in neuronal health during aging and may thus compromise protection against neurodegenerative diseases.

SIRT2 was expressed myelin in this study. SIRT2 is known to present in the cytoplasm of mature myelin sheath and Schwann cells,[24] which supports our findings. SIRT2 is expressed predominantly in oligodendrocytes, the myelin-producing cells of the central nervous system.[25] In contrast to other sirtuins, SIRT4 was expressed in vessels. SIRT5 was also expressed in vessels. It was known that SIRT4 is expressed in all tissues with highest levels in the kidney, heart, brain, and liver.[26] The role of SIRT4 in the vessels remains unknown. The expression pattern of SIRT5-7 was similar to SIRT1 and SIRT3 in that SIRT5-7 were expressed in neurons. SIRT5 localizes to the mitochondrial matrix, but also is identified in the intermembrane space. SIRT5 interacts with cytochrome c, a protein linked to mitochondrial metabolism and apoptotic signaling.[27] However, the significance of SIRT5 activity in metabolism is not yet known. The role of SIRT6 in brain function and neuronal survival is largely unknown. Nevertheless, SIRT6 may play a role in synaptic function and neuronal maturation and it

may be implicated in the regulation of neuronal survival.[28] Little is known about the role of SIRT7 in brain and neuronal cells.

Of note, no sirtuin was expressed in astrocytes, which are the most abundant cells in the brain. Considering that each sirtuin was mainly expressed in one cell type, such as SIRT1, SIRT3, and SIRT5-7 in neurons, SIRT in myelin, and SIRT4 in vessels, each sirtuin may serve their specific functions in the brain.

We examined the expression pattern of sirtuins after induction of cerebral ischemia, which is one of the most severe types of brain injuries. Expressions of SIRT1, SIRT6 and SIRT7 showed decreased tendency in the ischemic brain. This is consistent with findings in previous studies.[15],[16],[29] The expression of SIRT1 was reduced in ischemic penumbra in the MCAO group. Hyperbaric oxygen preconditioning-induced ischemic tolerance against cerebral ischemic injury was associated with upregulation of SIRT1, which suggests that to role of SIRT1 against ischemic injury in neuroprotection.[15] The expression of SIRT6 also tended to be decreased in ischemic areas of the rat brain in this study. The levels of SIRT6 in neuron were susceptible to ischemia, and were associated with the release of high mobility group box-1 that participates in brain damage.[16] Little is known on the expression or role of SIRT7 in cerebral ischemia as well as in normal brain. However, SIRT7-deficient cardiomyocytes were more susceptible to oxidative and genotoxic stress.[29] Along with those previous studies, our findings suggest that SIRT7 may also have a role in protection during ischemic episodes.

In this study, the expressions of SIRT3 and SIRT5 tend to be increased in the ischemic region. Furthermore, the expression of SIRT4 was significantly increased in the ischemic area, suggesting that seven sirtuins might have different functions positively or negatively for the neuroprotection. SIRT4 has tumor-suppressive activity and regulates the cellular metabolic response to DNA damage by inhibiting glutamine metabolism.[30] Moreover, SIRT4 plays a protective role in hypoxia-induced apoptosis in H9c2 cardiomyoblast cells and it might be beneficial in the treatment of ischemic heart disease.[31] SIRT4 is induced in response to stress. These findings suggest that increase of SIRT4 in vessels might be related to the protective response to ischemic injury.[30]

This present study provided basic information of expression patterns and distributions of all sirtuin families in the whole brain of various rodents. This study also suggests that one should be cautious in choosing species/strain of rodent and interpreting the result in sirtuin-related researches in the brain because there were some differences in the expression pattern between different species/strains of

rodent. In addition, the expression was quite different among sirtuins in the ischemic brain. These findings suggest the different role of each sirtuin in pathologic conditions in the brain.

V. CONCLUSION

Distribution and cellular localization in the rodent brain were different among sirtuins. Expression in the ischemic brain was also different. Findings of this study might provide with basic information for studying pathophysiologic role of sirtuins in the brain.

REFERENCES

1. North BJ, Verdin E. Sirtuins: Sir2-related NAD-dependent protein deacetylases. Genom e Biol 2004;5:224.
2. Morris KC, Lin HW, Thompson JW, Perez-Pinzon MA. Pathways for ischemic cytopro tection: role of sirtuins in caloric restriction, resveratrol, and ischemic preconditioning. J Cereb Blood Flow Metab 2011;31:1003-19.
3. Michishita E, Park JY, Burneskis JM, Barrett JC, Horikawa I. Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. Mol B iol Cell 2005;16:4623-35.
4. Ahn BH, Kim HS, Song S, Lee IH, Liu J, Vassilopoulos A, et al. A role for the mit ochondrial deacetylase Sirt3 in regulating energy homeostasis. Proc Natl Acad Sci U S A 2008;105:14447-52.
5. Schwer B, Schumacher B, Lombard DB, Xiao C, Kurtev MV, Gao J, et al. Neural sir tuin 6 (Sirt6) ablation attenuates somatic growth and causes obesity. Proc Natl Acad S ci U S A 2010;107:21790-4.
6. Cooper HM, Spelbrink JN. The human SIRT3 protein deacetylase is exclusively mitoc hondrial. Biochem J 2008;411:279-85.
7. Du J, Zhou Y, Su X, Yu JJ, Khan S, Jiang H, et al. Sirt5 is a NAD-dependent protei n lysine demalonylase and desuccinylase. Science 2011;334:806-9.
8. Jiang H, Khan S, Wang Y, Charron G, He B, Sebastian C, et al. SIRT6 regulates TN F-alpha secretion through hydrolysis of long-chain fatty acyl lysine. Nature 2013;496:1 10-3.
9. Yamamoto H, Schoonjans K, Auwerx J. Sirtuin functions in health and disease. Mol Endocrinol 2007;21:1745-55.
10. Tang BL. Sirt1's complex roles in neuroprotection. Cell Mol Neurobiol 2009;29:1093-1 03.
11. Preyat N, Leo O. Sirtuin deacylases: a molecular link between metabolism and immun ity. J Leukoc Biol 2013;93:669-80.
12. Zhang F, Wang S, Gan L, Vosler PS, Gao Y, Zigmond MJ, et al. Protective effects a nd mechanisms of sirtuins in the nervous system. Prog Neurobiol 2011;95:373-95.
13. Donmez G. The neurobiology of sirtuins and their role in neurodegeneration. Trends P harmacol Sci 2012;33:494-501.
14. Raval AP, Dave KR, Perez-Pinzon MA. Resveratrol mimics ischemic preconditioning i n the brain. J Cereb Blood Flow Metab 2006;26:1141-7.
15. Yan W, Fang Z, Yang Q, Dong H, Lu Y, Lei C, et al. SirT1 mediates hyperbaric ox

ygen preconditioning-induced ischemic tolerance in rat brain. J Cereb Blood Flow Metab 2013;33:396-406.

16. Lee OH, Kim J, Kim JM, Lee H, Kim EH, Bae SK, et al. Decreased expression of sirtuin 6 is associated with release of high mobility group box-1 after cerebral ischemia. Biochem Biophys Res Commun 2013;438:388-94.
17. Nie H, Chen H, Han J, Hong Y, Ma Y, Xia W, et al. Silencing of SIRT2 induces cell death and a decrease in the intracellular ATP level of PC12 cells. Int J Physiol Pathophysiol Pharmacol 2011;3:65-70.
18. Cakir I, Perello M, Lansari O, Messier NJ, Vaslet CA, Nillni EA. Hypothalamic Sirt1 regulates food intake in a rodent model system. PLoS One 2009;4:e8322.
19. Sidorova-Darmos E, Wither RG, Shulyakova N, Fisher C, Ratnam M, Aarts M, et al. Differential expression of sirtuin family members in the developing, adult, and aged rat brain. Front Aging Neurosci 2014;6:333.
20. Sundaresan NR, Samant SA, Pillai VB, Rajamohan SB, Gupta MP. SIRT3 is a stress-responsive deacetylase in cardiomyocytes that protects cells from stress-mediated cell death by deacetylation of Ku70. Mol Cell Biol 2008;28:6384-401.
21. Kim HS, Patel K, Muldoon-Jacobs K, Bisht KS, Aykin-Burns N, Pennington JD, et al. SIRT3 is a mitochondria-localized tumor suppressor required for maintenance of mitochondrial integrity and metabolism during stress. Cancer Cell 2010;17:41-52.
22. Herskovits AZ, Guarente L. SIRT1 in neurodevelopment and brain senescence. Neuron 2014;81:471-83.
23. Someya S, Yu W, Hallows WC, Xu J, Vann JM, Leeuwenburgh C, et al. Sirt3 mediates reduction of oxidative damage and prevention of age-related hearing loss under caloric restriction. Cell 2010;143:802-12.
24. Michan S, Sinclair D. Sirtuins in mammals: insights into their biological function. Biochem J 2007;404:1-13.
25. Werner HB, Kuhlmann K, Shen S, Uecker M, Schardt A, Dimova K, et al. Proteolipid protein is required for transport of sirtuin 2 into CNS myelin. J Neurosci 2007;27:7717-30.
26. Haigis MC, Mostoslavsky R, Haigis KM, Fahie K, Christodoulou DC, Murphy AJ, et al. SIRT4 inhibits glutamate dehydrogenase and opposes the effects of calorie restriction in pancreatic beta cells. Cell 2006;126:941-54.
27. Schlicker C, Gertz M, Papatheodorou P, Kachholz B, Becker CF, Steegborn C. Substrates and regulation mechanisms for the human mitochondrial sirtuins Sirt3 and Sirt5. J Mol Biol 2008;382:790-801.
28. Cardinale A, de Stefano MC, Mollinari C, Racaniello M, Garaci E, Merlo D. Bioche

mical characterization of sirtuin 6 in the brain and its involvement in oxidative stress response. Neurochem Res 2015;40:59-69.

29. Vakhrusheva O, Smolka C, Gajawada P, Kostin S, Boettger T, Kubin T, et al. Sirt7 increases stress resistance of cardiomyocytes and prevents apoptosis and inflammatory cardiomyopathy in mice. Circ Res 2008;102:703-10.

30. Jeong SM, Xiao C, Finley LW, Lahusen T, Souza AL, Pierce K, et al. SIRT4 has tumor-suppressive activity and regulates the cellular metabolic response to DNA damage by inhibiting mitochondrial glutamine metabolism. Cancer Cell 2013;23:450-63.

31. Liu B, Che W, Xue J, Zheng C, Tang K, Zhang J, et al. SIRT4 prevents hypoxia-induced apoptosis in H9c2 cardiomyoblast cells. Cell Physiol Biochem 2013;32:655-62.

ABSTRACT (IN KOREAN)

쥐의 정상 및 허혈 뇌에서의 sirtuin의 발현과 분포

< 지도교수 허지회 >

연세대학교 대학원 의과학과

김 자 영

Sirtuins (SIRTs) 은 니코틴아미드 아데닌 디뉴클레오티드에 의존적인 히스톤 탈아세틸화 효소이다. 지금까지 SIRT1부터 SIRT7까지 7개의 sirtuins이 알려져 있다. 정상 뇌와 병리학적인 뇌에서의 sirtuins의 역할을 이해하기 위해서는 뇌에서의 sirtuins의 해부학적 위치와 세포 내 발현에 대한 기본적인 지식이 필요하다. 그럼에도 불구하고 단지 제한된 정보만 존재해왔다. 우리는 설치류의 정상 뇌와 허혈성 뇌에서 sirtuins의 세포 내 위치와 분포를 알아보았다.

Sprague-Dawley (SD) 흰쥐, Wistar 흰쥐, Imprinting Control Region (ICR) 쥐, 그리고 C57BL/6 쥐를 사용했다. 정상 뇌의 전체 표본은 마취한 상태에서 심장 관류를 통해 얻었다. 뇌경색은 나일론 사를 이용하여 2시간 동안의 중대뇌동맥 폐색과 22시간의 재관류를 통해 유도된다. 파라핀이나 동결 절편은 면역조직화학법을 진행했고 전체 뇌는 크게, 후신경구, 대뇌, 소뇌로 나누어 관찰하였다. Sirtuins을 발현하는 세포 타입을 알기 위해 각 sirtuin과 특정 세포 마커인 항체로 이중 형광 염색했다. 신경세포에는 NeuN이나 β-III tubulin, 수초에는 미엘린 기본 단백질 (MBP), 희돌기교세포에는 CNPase, 성상세포에는 아교 섬유 산성 단백질 (GFAP), 그리고 혈관에는 collagen type IV나 RECA-1를 사

용하였다.

SIRT1, SIRT3 그리고 SIRT5-7 은 대부분의 부위의 신경세포에서 발현되었고 뇌량과 내포의 희돌기교세포에서도 발현되었다. SIRT2는 수초에서 발현되었고 SIRT4는 혈관에서 발현되었다. 결과적으로, SIRT5 - 7 는 후 신경구, 대뇌, 그리고 소뇌에 걸쳐 넓게 분포하고 높게 발현되었다. SIRT1 과 SIRT3 는 뇌량과 내포에서 종에 따라서는 발현을 보기 힘들었다. SIRT2는 설치류 뇌의 대부분의 부위에서 높게 발현됐지만, 후 신경구의 외부층 상층과 대뇌의 시상하부에서는 그렇지 않았다. SIRT3 역시 폭넓게 분포했지만 후 신경구의 전방 후각 핵과 소뇌의 분자 층에서는 발현 정도가 인식하기에 충분치 않았다. SIRT4는 소뇌의 퍼킨지 층을 제외하고는 전체 뇌에 넓게 분포했다. SIRT5 - 7 은 선택한 모든 부위에서 전체적으로 발현되었다. 뇌경색을 유도한 후에는, SIRT4 의 경우 뇌경색이 온 부위에서 상당히 증가된 발현을 보였다. 하지만, 다른 sirtuin들은 크게 다르지 않았다.

설치류의 뇌에서의 분포와 세포 내 위치는 각각의 sirtuins 마다 다르게 나타났다. 허혈성 뇌에서의 발현 또한 달랐다. 이러한 연구는 sirtuins이 뇌에서의 병리학적 역할을 연구하는 데 있어서 기본적인 정보를 제공할 것이다.

핵심되는 말 : sirtuin, 뇌, 분포, 면역조직화학법, 설치류